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Development of High-Resolution Scanning Electrochemical Microscopy for Nanoscale Topography and Electrochemical Simultaneous Imaging

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ABSTRACT

This article reviews our recent progress in the development of high-resolution scanning electrochemical microscopy (SECM) and its application to biological samples. SECM uses an ultramicroelectrode (UME) as a probe and a scanning mechanical stage for controlling the probe position. To improve the resolution of SECM, we have developed a fabrication method for pyrolytic carbon nanoelectrodes and a current feedback system for probe–sample distance control. The current feedback system effectively provides high-quality electrochemical and non-contact topography images because the current signal depends on the probe–sample distance. High-resolution SECM has overcome the limit of microscale imaging resolution and enabled the imaging of local regions within cells. In this study, we address four topics: nanoelectrode fabrication, current feedback probe–sample distance control systems, membrane protein imaging, and neurotransmitter detection.

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1. Introduction

Scanning electrochemical microscopy (SECM) is a probe microscopy technique in which an ultramicroelectrode (UME) is used as a probe.^{1,2} Electrode miniaturization allows localized space measurement and offers several advantages owing to the physical effects associated with length, such as low double-layer charging currents, low ohmic drops, fast mass transport, and diffusion rate-limiting steady-state current. The steady-state current of the voltammogram of the UME is described as follows:^{3,4}

$$i = 4nFDCa \quad (1)$$

where F is Faraday's constant; D and C are the diffusion coefficient and bulk concentration of the redox species, respectively; and a is the radius of the UME. SECM has been used to characterize and image the local electrochemical properties of various materials by scanning the sample surfaces with a UME. Furthermore, SECM has been used to monitor short-life chemicals,⁵ local catalytic reactivities,⁶ fast mass transport,⁷ and neurotransmitters.⁸ A particularly useful application of SECM is the observation of the respiration activity of live cells because of its non-invasive nature. Drug sensitivity assays and single embryo activity evaluations have been performed using SECM.^{9,10}

The spatial and temporal resolutions of SECM depend primarily on the tip size of the UME and the distance between the UME and the sample. However, nanoscale electrochemical imaging by SECM remains a challenge because of the difficulty in miniaturizing the UME. Significant efforts have been made to improve the SECM resolution. Distance control is also important for improving the SECM resolution. Here, we present an overview of our recent progress in the development of high-resolution SECM for biological sample imaging.

2. Instrumentation

Figure 1 shows a schematic of the high-resolution SECM setup. SECM instrumentation has been described in detail in previous papers.^{11–13} The SECM system comprises six major components: an electrochemical cell (including a microelectrode and a reference electrode), a current detector (Axon Instruments, MultiClamp 700B), a piezo scanner (Nanocontrol, 621.2CL and 621.ZCL), a data acquisition and control system (National Instruments, PCIe-7841R), a vibration-free table (Herz, TS140), and a homemade software program written in LabVIEW 2014 (National Instruments). The sensitivity and time response of the current detector are important when using a current signal for distance control. We set the frequency of the low-pass filter as 1–3 kHz for detecting the

Figure 1. (Color online) Block diagram of the SECM measurement.

current response. The piezo scanner is crucial for obtaining high-resolution SECM images because it enables stable and accurate positioning. The vibration-free table provides adequate vibration isolation for high-resolution measurements.

3. Nanoelectrode Fabrication

The reliable fabrication of nanoelectrodes is important for improving the spatial resolution of SECM. In particular, various approaches have been adopted to create small electrodes with thin insulating coatings, such as photolithography, chemical vapor deposition, electrodeposition, laser pulling techniques, and pyrolytic carbon deposition. Mezour and coworkers recently developed a reproducible procedure for the fabrication of Pt disk-shaped microelectrodes with diameters ranging from 50 nm to 1 μ m using a laser pulling technique.¹⁴ We developed a fabrication technique based on pyrolytic carbon deposition inside glass pipettes that enables the fabrication of nanoelectrodes with insulation of excellent integrity.^{12,13} To fabricate the pyrolytic carbon electrode, a quartz glass capillary (O.D. 1.2 mm, I.D. 0.9 mm; Sutter Instruments, USA) was pulled using a CO₂ laser puller (Sutter Instruments, USA, model P-2000,). Butane gas was passed through the quartz capillary using a Tygon tube (O.D. 2.4 mm, I.D. 0.8 mm). The pipette was internally pressurized with butane to deposit the carbon. The taper of the pipette was inserted into another quartz capillary (O.D. 1.0 mm, I.D. 0.7 mm; Sutter Instruments, USA), which was filled with argon gas to prevent oxidation of the carbon layer and bending of the capillary at high temperatures. This approach also protected the pipette aperture from closing due to the softening of the quartz pipette walls. To form a pyrolytic carbon layer inside the capillary, the pipette taper was then heated with a Bunsen burner for durations ranging from 0.5 s for a 100-nm-radius electrode to 3 s for a 1- μ m-radius electrode.

4. Electrode-sample Distance Control

Because the probe-sample distance affects the diffusion state of the molecules around the electrode, precise control of this distance is critical for both topography imaging and high-quality electrochemical imaging. Specifically, topography-related artifacts in these images can be avoided by employing probe-sample distance control. Various feedback distance control systems have been developed, including those based on atomic force microscopy,^{15,16} shear force,^{17–19} impedance,^{20–22} faradaic current,^{20,23} ion current,^{11,12,24} and electrochemical signals.^{25–27} Impedance-feedback and constant-current distance control systems are effective because additional modification of the probe for distance control is not required. The impedance can be simply expressed as a series combination of the solution resistance and double-layer capacitance. The double-layer capacitance, which varies with the probe-sample distance, is used as a feedback signal. Therefore, the ratio of capacitive reactance and solution resistance is important. However, the impedance-feedback mode has resolution limitations because the electrode size decreases with decrease in the ratio of capacitive reactance and solution resistance.²⁰ The constant faradaic current mode has been shown to be capable of high-resolution topography imaging.²³ But it is impossible to get faradaic current image simultaneously because faradaic current signal is used as a feedback signal.

Schuhmann and coworkers reported the first shear-force feedback SECM. Shear force can be detected using two methods: a tuning fork method and an optical method. The tuning fork method has become the standard approach for controlling the probe position with a relatively simple and low-cost design.¹⁷ Therefore, we developed a tuning-fork-based distance control SECM and successfully imaged the topography respiration activity of a single cell.¹⁸ The probe was attached to one of the legs of a tuning fork and vibrated using a piezoelectric buzzer to drive the tuning fork into the

resonance state. When the probe was positioned away from the substrate, the vibration from the buzzer induced a voltage signal in the tuning fork due to the piezoelectric effect. As the distance between the probe and the substrate became less than 100 nm, the shear force between them decreased the magnitude of the vibration of the tuning fork. To control the probe-sample distance by using this amplitude signal as a feedback signal. For successfully imaging living cell topographies, we optimized the operating conditions of the vibrating probe to detect the shear forces between the probe and the living cells.¹⁷

Unwin and coworkers reported scanning electrochemical cell microscopy (SECCM).^{28–30} A solution-filled nanopipette is used as a probe to investigate the sample surface reactivity under an atmospheric environment. The sample is connected to the working electrode, and the reference electrode is inserted into the nanopipette. SECCM is useful for nanoscale electrochemical imaging because the measurement space is isolated by the local electrochemical cell and the nanopipette is easy to fabricate. Previously, SECCM was used to evaluate the reactivity of highly oriented pyrolytic graphite,³¹ carbon nanotubes,²⁶ nanoparticles,²⁵ and Pt of different orientations.^{32,33} We applied SECCM to the cathode material of lithium ion secondary batteries.³⁴ SECCM is effective for material research but is difficult for live cell imaging because the measurements must be performed under ambient conditions.

Scanning ion conductance microscopy (SICM) is a promising technique for probe-sample distance control under physiological conditions.^{35–40} SICM uses a nanopipette as a scanning probe for detecting the ionic current between an electrode inside the pipette and an electrode in a bath. When the distance between the pipette and the sample is reduced, the ion flow at the top of the nanopipette is inhibited and the ionic current decreases. The pipette-sample distance is then controlled on the basis of this current change.

We have developed a combined SECM-SICM probe, which has the dual functionality of a nanopipette and nanoelectrode, to study the topography and chemical release of living cells in a physiological environment.^{11,12,41} SECM-SICM is effective for not only topography imaging but also electrochemical measurements.^{24,42–44} For example, we imaged glucose oxidase spots using SECM-SICM. Figure 2 shows SICM topographic and SECM images in which the probe-sample distances were held at 100 and 600 nm. The SICM image revealed the highly resolved structure of the glucose oxidase spot surface with 320 nm $\times$ 1600 nm small caves. The SECM image at 100 nm also shows this fine structure with small caves; however, the cave-like structure is not observed at 600 nm. These results clearly demonstrate that SICM distance regulation is very effective for improving the resolution for electrochemical imaging.

We have also developed a simple and effective constant-distance mode SECM technique called voltage-switching mode (VSM)-SECM.¹³ VSM-SECM does not require additional modification of the probe as it achieves distance control by switching the applied voltage of the working electrode. As the electrode is moved toward the sample surface, the distance-dependent current for the hindered diffusion of a redox-active solute to the tip is monitored. When the electrode reaches the desired position at each point, the applied voltage can be switched to a positive value for electrochemical (flux) imaging of the sample surface. The time required to reach the steady-state current is dramatically shorter for a nanoelectrode than it is for a combinational UME because the nanoelectrode forms a hemi-diffusion layer quickly and effectively.

Whole-cell imaging via scanning probe microscopy is still challenging because of the steep slope of the cell topography. The development of scanning protocols is also important for imaging the topography of an entire live cell. The hopping mode, in which the probe repetitively approaches the sample surface and retracts at each point, is a promising method for imaging steep slope surface topographies.^{11–13,36} However, the application of the hopping mode

Figure 2. (A and D) Topographic and (B, C, E, and F) electrochemical images of a glucose oxidase immobilized substrate adding 20 mM glucose in 0.50 mM FcCH_2OH + 0.1 M KCl. The SECM nanoring and SICM nanopipette electrodes were held at 500 and 200 mV vs. Ag/AgCl, respectively. Upper and lower images were captured with $8\ \mu\text{m} \times 8\ \mu\text{m}$ and $2\ \mu\text{m} \times 2\ \mu\text{m}$, respectively. The probe-sample distances were held at 100 nm (B and E) and 600 nm (C and F), respectively. (Reprinted with permission from Ref. Takahashi et al., Copyright 2010 ACS.)

Figure 3. (Color online) (a) Schematic illustration of EGFR detection using SECM. (b) Single CHO cell SECM image of EGFR/CHO cell and normal CHO cell. (Reprinted with permission from Ref. Takahashi et al., Copyright 2009 ACS.)

is limited by its temporal resolution as the hopping process is more time-consuming than conventional lateral scanning methods. This limitation was resolved by changing the step size during measurement and prescanning the area of interest.³⁶

5. Membrane Protein Imaging

We have developed a method for imaging cell membrane proteins using SECM. To image specific membrane proteins, we labeled the membrane proteins with enzymes via an antibody. The chemical produced by the enzyme-catalyzed reaction was then detected by SECM. The measurement of membrane proteins by SECM has several advantages. First, a single adherent cell can be measured without removing it from the culture dish. Second, the antibody concentration can be optimized because faradaic current is suitable for quantitative estimation. Third, a faradaic current image corresponding to the expression state of the measured membrane protein is available at the single-cell level. Finally, the change of the

membrane expression level of the apical side during internalization, a region that is difficult to image using total internal reflection fluorescence microscopy, can be evaluated by using electrochemical measurements to detect the diffused chemicals in a solution. We characterized the expression level of the epidermal growth factor receptor (EGFR) by SECM.⁴⁵ EGFR is a key membrane protein associated with cancer. To estimate the EGFR expression levels by SECM, EGFR was labeled with alkaline phosphatase (ALP) via an antibody (Fig. 3). The oxidation current of *p*-aminophenol (PAP) produced by the ALP-catalyzed reaction was monitored to estimate the density of EGFR on the cell surface. The decrease in the expression level of EGFR induced by Epidermal Growth Factor (EGF)-triggered endocytosis was estimated by comparing the faradaic current responses of the cells with and without EGF stimulation. Figure 3 shows SECM images of a single EGFR-expressing Chinese Hamster Ovary (CHO) cell and a wild-type CHO cell. The genetically engineered CHO shows high electrochemical signals, indicating that EGFR is expressed at the cell

surface. We optimized the concentration of the labeling antibody for EGFR at the cell surface and confirmed distinct differences in EGFR expression levels among different cell types. The SECM results were found to be comparable to the results of flow cytometry experiments.

We have also reported an SECM-based receptor-mediated endocytosis detection method.⁴⁶ Receptor-mediated endocytosis is an important process that negatively regulates receptor-mediated signals by reducing the surface concentration of the receptor itself (downregulation) and controls the strength and duration of the signals downstream. The receptor is then recycled into the plasma membrane. EGF binds to EGFR, and the activated EGFR initiates the signaling cascades, thereby promoting cell proliferation, differentiation, apoptosis, and migration. This signaling is controlled by EGF-triggered endocytosis, which reduces the number of EGFR molecules exposed to the outside medium. Because SECM was used to detect the ALP activity on the outer membrane, this procedure helped discriminate the EGFR on the outer membrane from the intracellular EGFR involved in endocytosis. SECM showed a marked decrease (93%) in the current responses generated owing to the AP activity upon the addition of EGF, clearly indicating that EGF triggered the endocytosis, leading to the withdrawal of most EGFRs from the outer membrane.

6. Neurotransmitter Detection

SECM is effective for investigating the spatial distribution of neurotransmitter release and for quantitatively analyzing single vesicles. Wightman and coworkers developed a neurotransmitter detection method using a carbon electrode and performed *in vivo* measurements.⁴⁷ This method enables the quantification of neurotransmitters from a single neural vesicle as a transient current spike in the electrode response.^{48,49} Schuhmann and coworkers detected the neurotransmitters using shear-force distance control SECM.⁵⁰ Liebetrau and coworkers imaged differentiated and undifferentiated rat adrenal gland pheochromocytoma (PC12) cell topographies using several mediators selected for their biocompatibility from a large pool of candidates.⁵¹ They also performed topography imaging of differentiated and undifferentiated PC12 cells using the constant-current mode or constant-impedance mode.²⁰ Because the neurites are thin compared to the cell height, they are difficult to image using the constant-height mode. The constant-impedance mode was applied to image the differentiated PC12 cells. An advantage of the impedance-based constant-distance mode is that the images can be recorded in the growth media without an added mediator, facilitating long-term imaging during growth and development. By combining amperometry and constant-height impedance, SECM has enabled the simultaneous mapping of topography and the detection of vesicular release events while moving the tip across a cell.

We have developed SECM–SICM¹² and VSM–SECM,¹³ both of which have visualized differentiated PC12 topography and detected neurotransmitter release events. Figure 4 shows SICM topography images of differentiated PC12 obtained using an SECM–SICM probe and a conventional SICM probe. A neurite with a width of approximately 100 nm can be visualized. Thus, the topography image obtained by our SICM–SECM probe is beyond the limit of optical microscopy. An advantage of SECM–SICM is that the electrolyte-filled probe can be used to apply different reagents for the local stimulation of a cell. Therefore, the voltage-driven application of K^+ ions was realized by SECM–SICM to achieve both the local depolarization of the cell membrane and simultaneous detection of the neurotransmitter. Under local stimulation, we detected current spikes at a lower frequency than in the case of entire-cell stimulation. This finding suggests that SECM–SICM can induce and detect localized releases of neurotransmitters over the cell surface.

Figure 4. (Color online) (a) Nanoscale topography images of differentiated PC12 cells using SECM–SICM. The arrows showed the dendritic structures. A series of current spikes corresponding to neurotransmitter release detected after (b) whole cell stimulation with 105 mM K^+ using another micropipette and (c) voltage-driven delivery of K^+ ions using a SICM barrel. (Reprinted with permission from Ref. Takahashi et al., Copyright 2011 John Wiley and Sons.)

7. Conclusion

We have developed a fabrication method for nanoelectrodes and electrode–sample distance control systems to improve the resolution of SECM. SECM is an effective tool for *in vitro* and *in vivo* measurements of chemicals. We have described impressive studies using SECM to examine biological molecules, such as the evaluation of membrane proteins and detection of neurotransmitters. The presented nanoelectrode and SICM distance control will open up new possibilities for the SECM measurement of live cells. In the future, SECM will reveal heterogeneous distributions of chemicals at the cell surface and intracellular chemical reaction profiles, allowing the cell local function to be linked to chemical information.

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References

1. A. J. Bard, F. R. F. Fan, J. Kwak, and O. Lev, *Anal. Chem.*, **61**, 132 (1989).
2. R. C. Engstrom and C. M. Pharr, *Anal. Chem.*, **61**, 1099A (1989).
3. J. L. Amphlett and G. Denuault, *J. Phys. Chem. B*, **102**, 9946 (1998).
4. Y. H. Shao and M. V. Mirkin, *J. Phys. Chem. B*, **102**, 9915 (1998).
5. F. H. Cao, J. Kim, and A. J. Bard, *J. Am. Chem. Soc.*, **136**, 18163 (2014).
6. J. L. Fernandez, D. A. Walsh, and A. J. Bard, *J. Am. Chem. Soc.*, **127**, 357 (2005).
7. Y. H. Shao, M. V. Mirkin, G. Fish, S. Kokotov, D. Palanker, and A. Lewis, *Anal. Chem.*, **69**, 1627 (1997).
8. R. M. Wightman, J. A. Jankowski, R. T. Kennedy, K. T. Kawagoe, T. J. Schroeder, D. J. Leszczyszyn, J. A. Near, E. J. Diliberto, and O. H. Viveros, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10754 (1991).
9. H. Shiku, T. Shiraishi, H. Ohya, T. Matsue, H. Abe, H. Hoshi, and M. Kobayashi, *Anal. Chem.*, **73**, 3751 (2001).
10. Y. S. Torisawa, T. Kaya, Y. Takii, D. Oyamatsu, M. Nishizawa, and T. Matsue, *Anal. Chem.*, **75**, 2154 (2003).
11. Y. Takahashi, A. I. Shevchuk, P. Novak, Y. Murakami, H. Shiku, Y. E. Korchev, and T. Matsue, *J. Am. Chem. Soc.*, **132**, 10118 (2010).
12. Y. Takahashi, A. I. Shevchuk, P. Novak, Y. J. Zhang, N. Ebejer, J. V. Macpherson, P. R. Unwin, A. J. Pollard, D. Roy, C. A. Clifford, H. Shiku, T. Matsue, D. Klenerman, and Y. E. Korchev, *Angew. Chem., Int. Ed.*, **50**, 9638 (2011).
13. Y. Takahashi, A. I. Shevchuk, P. Novak, B. Babakinejad, J. Macpherson, P. R. Unwin, H. Shiku, J. Gorelik, D. Klenerman, Y. E. Korchev, and T. Matsue, *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 11540 (2012).
14. M. A. Mezour, M. Morin, and J. Mauzeroll, *Anal. Chem.*, **83**, 2378 (2011).
15. J. V. Macpherson and P. R. Unwin, *Anal. Chem.*, **72**, 276 (2000).
16. C. Kranz, G. Friedbacher, and B. Mizaikoff, *Anal. Chem.*, **73**, 2491 (2001).
17. Y. Takahashi, H. Shiku, T. Murata, T. Yasukawa, and T. Matsue, *Anal. Chem.*, **81**, 9674 (2009).
18. Y. Takahashi, Y. Hirano, T. Yasukawa, H. Shiku, H. Yamada, and T. Matsue, *Langmuir*, **22**, 10299 (2006).
19. A. Hengstenberg, C. Kranz, and W. Schuhmann, *Chem. Eur. J.*, **6**, 1547 (2000).
20. R. T. Kurulugama, D. O. Wipf, S. A. Takacs, S. Pongmayteegul, P. A. Garriss, and J. E. Baur, *Anal. Chem.*, **77**, 1111 (2005).
21. X. C. Zhao, P. M. Diakowski, and Z. F. Ding, *Anal. Chem.*, **82**, 8371 (2010).
22. M. A. Alpuche-Aviles and D. O. Wipf, *Anal. Chem.*, **73**, 4873 (2001).
23. F. R. F. Fan and A. J. Bard, *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 14222 (1999).
24. D. J. Comstock, J. W. Elam, M. J. Pellin, and M. C. Hersam, *Anal. Chem.*, **82**, 1270 (2010).
25. S. C. S. Lai, P. V. Dudin, J. V. Macpherson, and P. R. Unwin, *J. Am. Chem. Soc.*, **133**, 10744 (2011).
26. A. G. Guell, N. Ebejer, M. E. Snowden, K. McKelvey, J. V. Macpherson, and P. R. Unwin, *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 11487 (2012).
27. C. G. Williams, M. A. Edwards, A. L. Colley, J. V. Macpherson, and P. R. Unwin, *Anal. Chem.*, **81**, 2486 (2009).
28. N. Ebejer, M. Schnippering, A. W. Colburn, M. A. Edwards, and P. R. Unwin, *Anal. Chem.*, **82**, 9141 (2010).
29. N. Ebejer, A. G. Guell, S. C. S. Lai, K. McKelvey, M. E. Snowden, and P. R. Unwin, *Annual Review of Analytical Chemistry*, **6**, 329 (2013).
30. M. E. Snowden, A. G. Guell, S. C. S. Lai, K. McKelvey, N. Ebejer, M. A. O'Connell, A. W. Colburn, and P. R. Unwin, *Anal. Chem.*, **84**, 2483 (2012).
31. A. G. Guell, N. Ebejer, M. E. Snowden, J. V. Macpherson, and P. R. Unwin, *J. Am. Chem. Soc.*, **134**, 7258 (2012).
32. B. D. B. Aaronson, C. H. Chen, H. J. Li, M. T. M. Koper, S. C. S. Lai, and P. R. Unwin, *J. Am. Chem. Soc.*, **135**, 3873 (2013).
33. C. H. Chen, K. E. Meadows, A. Cuharuc, S. C. S. Lai, and P. R. Unwin, *PCCP*, **16**, 18545 (2014).
34. Y. Takahashi, A. Kumtani, H. Munakata, H. Inomata, K. Ito, K. Ino, H. Shiku, P. R. Unwin, Y. E. Korchev, K. Kanamura, and T. Matsue, *Nat. Commun.*, **5** (2014).
35. Y. E. Korchev, C. L. Bashford, M. Milovanovic, I. Vodyanov, and M. J. Lab, *Biophys. J.*, **73**, 653 (1997).
36. P. Novak, C. Li, A. I. Shevchuk, R. Stepanyan, M. Caldwell, S. Hughes, T. G. Smart, J. Gorelik, V. P. Ostanin, M. J. Lab, G. W. J. Moss, G. I. Frolenkov, D. Klenerman, and Y. E. Korchev, *Nat. Methods*, **6**, 279 (2009).
37. A. I. Shevchuk, G. I. Frolenkov, D. Sanchez, P. S. James, N. Freedman, M. J. Lab, R. Jones, D. Klenerman, and Y. E. Korchev, *Angew. Chem., Int. Ed.*, **45**, 2212 (2006).
38. Y. Takahashi, Y. Murakami, K. Nagamine, H. Shiku, S. Aoyagi, T. Yasukawa, M. Kanzaki, and T. Matsue, *PCCP*, **12**, 10012 (2010).
39. Y. Takahashi, K. Ito, X. W. Wang, Y. Matsumae, H. Komaki, A. Kumtani, K. Ino, H. Shiku, and T. Matsue, *Electrochemistry*, **82**, 331 (2014).
40. Y. Nashimoto, Y. Takahashi, H. Ida, Y. Matsumae, K. Ino, H. Shiku, and T. Matsue, *Anal. Chem.*, **87**, 2542 (2015).
41. M. Sen, Y. Takahashi, Y. Matsumae, Y. Horiguchi, A. Kumtani, K. Ino, H. Shiku, and T. Matsue, *Anal. Chem.*, **87**, 3484 (2015).
42. M. A. O'Connell, J. R. Lewis, and A. J. Wain, *Chem. Commun.*, **51**, 10314 (2015).
43. M. A. O'Connell and A. J. Wain, *Anal. Chem.*, **86**, 12100 (2014).
44. C. A. Morris, C. C. Chen, and L. A. Baker, *Analyst*, **137**, 2933 (2012).
45. Y. Takahashi, T. Miyamoto, H. Shiku, R. Asano, T. Yasukawa, I. Kumagai, and T. Matsue, *Anal. Chem.*, **81**, 2785 (2009).
46. Y. Takahashi, T. Miyamoto, H. Shiku, K. Ino, T. Yasukawa, R. Asano, I. Kumagai, and T. Matsue, *PCCP*, **13**, 16569 (2011).
47. P. E. M. Phillips, G. D. Stuber, M. L. A. V. Heien, R. M. Wightman, and R. M. Carelli, *Nature*, **422**, 614 (2003).
48. T. K. Chen, G. O. Luo, and A. G. Ewing, *Anal. Chem.*, **66**, 3031 (1994).
49. K. P. Troyer, M. L. A. V. Heien, B. J. Venton, and R. M. Wightman, *Curr. Opin. Chem. Biol.*, **6**, 696 (2002).
50. A. Hengstenberg, A. Blochl, I. D. Dietzel, and W. Schuhmann, *Angew. Chem., Int. Ed.*, **40**, 905 (2001).
51. J. M. Liebetrau, H. M. Miller, and J. E. Baur, *Anal. Chem.*, **75**, 563 (2003).